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(54) Title: METHOD AND MEDICAMENT FOR INHIBITING THE EXPRESSION OF A DEFINED GENE

(54) Bezeichnung: VERFAHREN UND MEDIKAMENT ZUR HEMMUNG DER EXPRESSION EINES VORGEGEBENEN GENS

(57) Abstract

The invention relates to a medicament containing at least one double-stranded oligoribonucleotide (dsRNA) designed to inhibit the expression of a target gene. According to the invention, one strand of the dsRNA is at least in part complementary to the target gene.

(57) Zusammenfassung

Die Erfindung betrifft ein Medikament mit mindestens einem Oligoribonukleotid mit doppelsträniger Struktur (dsRNA) zur Hemmung der Expression eines Zielgens, wobei ein Strang der dsRNA zumindest abschnittsweise komplementär zum Zielgen ist.

Method and medicament for inhibiting the expression of a given gene

The invention relates to a method in accordance with the preamble of claim 1. It furthermore relates to a medicament and to a use of double-stranded oligoribonucleotides and to a vector encoding them.

Such a method is known from WO 99/32619, which was unpublished at the priority date of the present invention. The known process aims at inhibiting the expression of genes in cells of invertebrates. To this end, the double-stranded oligoribonucleotide must exhibit a sequence which is identical with the target gene and which has a length of at least 50 bases. To achieve efficient inhibition, the identical sequence must be 300 to 1 000 base pairs in length. Such an oligoribonucleotide is complicated to prepare.

DE 196 31 919 C2 describes an antisense RNA with specific secondary structures, the antisense RNA being present in the form of a vector encoding it. The antisense RNA takes the form of an RNA molecule which is complementary to regions of the mRNA. Inhibition of the gene expression is caused by binding to these regions. This inhibition can be employed in particular for the diagnosis and/or therapy of diseases, for example tumor diseases or viral infections. - The disadvantage is that the antisense RNA must be introduced into the cell in an amount which is at least as high as the amount of the mRNA. The known antisense methods are not particularly effective.

US 5,712,257 discloses a medicament comprising mismatched double-stranded RNA (dsRNA) and bioactive mismatched fragments of dsRNA in the form of a ternary complex together with a surfactant. The dsRNA used for this purpose consists of synthetic nucleic acid single strands without defined base sequence. The single

strands undergo irregular base pairing, also known as "non-Watson-Crick" base pairing, giving rise to mismatched double strands. The known dsRNA is used to inhibit the amplification of retroviruses such as HIV. Amplification of the virus can be inhibited when non-sequence-specific dsRNA is introduced into the cells. This leads to the induction of interferon, which is intended to inhibit viral amplification. The inhibitory effect, or the activity, of this method is poor.

It is known from Fire, A et al., NATURE, Vol. 391, pp. 806 that dsRNA whose one strand is complementary in segments to a nematode gene to be inhibited inhibits the expression of this gene highly efficiently. It is believed that the particular activity of the dsRNA used in nematode cells is not due to the antisense principle but possibly on catalytic properties of the dsRNA, or enzymes induced by it. - Nothing is mentioned in this paper on the activity of specific dsRNA with regard to inhibiting the gene expression, in particular in mammalian and human cells.

It would be advantageous if at least preferred embodiments of the invention were to do away with the disadvantages of the prior art. In particular, it would be advantageous if at least preferred embodiments of the invention provided as effective as possible a method, medicament or use for the preparation of a medicament, which method, medicament or use is capable of causing particularly effective inhibition of the expression of a given target gene.

In one aspect, the invention provides a method for inhibiting the expression of a given target gene in a cell, wherein an oligoribonucleotide with double-stranded structure (dsRNA) which has 15 to 49 base pairs is introduced into the cell, wherein one strand of the dsRNA has a region I with not more than 49 successive nucleotide pairs and which is at least in parts complementary to the

target gene and wherein a complementary region II within the double-stranded structure is formed by two separate RNA single strands.

In accordance with the present invention, the region I which is complementary to the target gene exhibits not more than 49 successive nucleotide pairs.

Provided in accordance with the invention are an oligoribonucleotide or a vector encoding therefor. At least segments of the oligoribonucleotide exhibit a defined nucleotide sequence. The defined segment may be limited to the complementary region I. However, it is also possible that all of the double-stranded oligoribonucleotide exhibits a defined nucleotide sequence.

Surprisingly, it has emerged that an effective inhibition of the expression of the target gene can be achieved even when the complementary region I is not more than 49 base pairs in length. The procedure of providing such oligoribonucleotides is less complicated.

In particular, dsRNA with a length of over 50 nucleotide pairs induces certain cellular mechanisms, for example the dsRNA-dependent protein kinase or the 2-5A system, in mammalian and human cells. This leads to the disappearance of the interference effect mediated by the dsRNA which exhibits a defined sequence. As a consequence, protein biosynthesis in the cell is blocked. The present invention overcomes this disadvantage in particular.

Furthermore, the uptake of dsRNA with short chain lengths into the cell or into the nucleus is facilitated markedly over longer-chain dsRNAs.

It has proved advantageous for the dsRNA or the vector to be present packaged into micellar structures, preferably in liposomes. The dsRNA or the vector can likewise be enclosed in viral natural capsids or in chemically or enzymatically produced artificial capsids or structures derived therefrom. - The abovementioned features make it possible to introduce the dsRNA or the vector into given target cells.

In accordance with the invention, the dsRNA has 15 to 49 base pairs. Thus, the dsRNA can be longer than the region I, which is complementary to the target gene. The complementary region I can be located at the terminus or inserted into the dsRNA. Such dsRNA or a vector provided for coding the same can be produced synthetically or enzymatically by customary methods.

The gene to be inhibited is expediently expressed in eukaryotic cells. The target gene can be selected from the following group: oncogene, cytokin gene, Id protein gene, developmental gene, prion gene. It can also be expressed in pathogenic organisms, preferably in plasmodia. It can be part of a virus or viroid which is preferably pathogenic to humans. - The method proposed makes it possible to produce compositions for the therapy of genetically determined diseases, for example cancer, viral diseases or Alzheimer's disease.

The virus or viroid can also be a virus or viroid which is pathogenic to animals or plant-pathogenic. In this case, the method according to the invention also permits the provision of compositions for treating animal or plant diseases.

In some embodiments, segments of the dsRNA are designed as double-stranded. A region II which is complementary within the double-stranded structure is formed by two separate RNA single strands or by autocomplementary regions of a topologically closed RNA single strand which is preferably in circular form.

The ends of the dsRNA can be modified to counteract degradation in the cell or dissociation into the single strands. Dissociation takes place in particular when low concentrations or short chain lengths are used. To inhibit dissociation in a particularly effective fashion, the cohesion of the complementary region II,

which is caused by the nucleotide pairs, can be increased by at least one, preferably two, further chemical linkage(s). - A dsRNA according to the invention whose dissociation is reduced exhibits greater stability to enzymatic and chemical degradation in the cell or in the organism.

The complementary region II can be formed by autocomplementary regions of an RNA hairpin loop, in particular when using a vector according to the invention. To afford protection from degradation, it is expedient for the nucleotides to be chemically modified in the loop region between the double-stranded structure.

The chemical linkage is expediently formed by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, preferably van-der-Waals or stacking interactions, or by metal-ion coordination. In an especially advantageous aspect, it can be formed at at least one, preferably both, end(s) of the complementary region II.

It has furthermore proved to be advantageous for the chemical linkage to be formed by one or more linkage groups, the linkage groups preferably being poly(oxyphosphinicooxy-1,3-propanediol) and/or poly-ethylene glycol chains. The chemical linkage can also be formed by purine analogs used in place of purines in the complementary regions II. It is also advantageous for the chemical linkage to be formed by azabenzene units introduced into the complementary regions II. Moreover, it can be formed by branched nucleotide analogs used in place of nucleotides in the complementary regions II.

It has proved expedient to use at least one of the following groups for generating the chemical linkage: methylene blue; bifunctional groups, preferably

bis(2-chloroethyl)amine; N-acetyl-N'-(p-glyoxyl-benzoyl)cystamine; 4-thiouracil; psoralene. The chemical linkage can furthermore be formed by thiophosphoryl groups provided at the ends of the double-stranded region. The chemical linkage at the ends of the double-stranded region is preferably formed by triple-helix bonds.

The chemical linkage can expediently be induced by ultraviolet light.

The nucleotides of the dsRNA can be modified. This counteracts the activation, in the cell, of a double-stranded-RNA-dependent protein kinase, PKR. Advantageously, at least one 2'-hydroxyl group of the nucleotides of the dsRNA in the complementary region II is replaced by a chemical group, preferably a 2'-amino or a 2'-methyl group. At least one nucleotide in at least one strand of the complementary region II can also be a locked nucleotide with a sugar ring which is chemically modified, preferably by a 2'-O, 4'-C methylene bridge. Advantageously, several nucleotides are locked nucleotides.

A further especially advantageous embodiment provides that the dsRNA or the vector is bound to, associated with or surrounded by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically. The coat protein can be derived from polyomavirus. The coat protein can contain the polyomavirus virus protein 1 (VP1) and/or virus protein 2 (VP2). The use of such coat proteins is known from, for example, DE 196 18 797 A1, whose disclosure is herewith incorporated. - The abovementioned features considerably facilitate the introduction of the dsRNA or of the vector into the cell.

When a capsid or capsid-type structure is formed from the coat protein, one side preferably faces the interior of the capsid or capsid-type structure. The construct formed is particularly stable.

The dsRNA can be complementary to the primary or processed RNA transcript of the target gene. - The cell can be a vertebrate cell or a human cell.

At least two dsRNAs which differ from each other or at least one vector encoding them can be introduced into the cell, where at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes. This makes it possible simultaneously to inhibit the expression of at least two different target genes. In order to suppress, in the cell, the expression of a double-stranded-RNA-dependent protein kinase, PKR, one of the target genes is advantageously the PKR gene. This allows effective suppression of the PKR activity in the cell.

In another aspect, the invention provides a medicament with at least one oligoribonucleotide with double-stranded structure (dsRNA) which contains 15 to 49 base pairs for inhibiting the expression of a given target gene in mammalian cells, wherein one strand of the dsRNA has a region I with not more than 49 successive nucleotide pairs and which is at least in parts complementary to the target gene and where a complementary region II within the double-stranded structure is formed by two separate RNA single strands. - Surprisingly, it has emerged that such a dsRNA is suitable as medicament for inhibiting the expression of a given gene in mammalian cells. In comparison with the use of single-stranded oligoribonucleotides, the inhibition is already caused at concentrations which are lower by at least one order of magnitude. The medicament according to the invention is

highly effective. Lesser side effects can be expected.

Also described herein is a medicament with at least one vector for coding at least one oligoribonucleotide with double-stranded structure (dsRNA) for inhibiting the expression of a given target gene, where one strand of the dsRNA has a region I where at least segments are complementary to the target gene. - The medicament proposed exhibits the abovementioned advantages. By using a vector, in particular production costs can be reduced.

The complementary region I has not more than 49 successive nucleotide pairs. Surprisingly, it has emerged that an effective inhibition of the expression of the target gene can be achieved even when the complementary region I is not more than 49 base pairs in length. The procedure of providing such oligoribonucleotides is less complicated.

In another aspect, the invention provides use of an oligoribonucleotide with double-stranded structure (dsRNA) which has 15 to 49 base pairs for the preparation of a medicament for inhibiting the expression of a given target gene in mammalian cells, wherein one strand of the dsRNA has a region I with not more than 49 successive nucleotide pairs and which is at least in parts complementary to the target gene and wherein a complementary region II within the double-stranded structure is formed by two separate RNA single strands. - Surprisingly, such a dsRNA is suitable for preparing a medicament for inhibiting the expression of a given gene. Compared with the use of single-stranded oligoribonucleotides, the inhibition is already caused at concentrations which are lower by one order of magnitude when using dsRNA. The use according to the invention thus makes possible the preparation of particularly effective medicaments.

Also described herein is the use of a vector for coding at least one oligoribonucleotide with double-stranded structure (dsRNA) for preparing a medicament for inhibiting the expression of a given target gene, where one strand of the dsRNA has a region I where at least segments are complementary to this target gene. - The use of a vector makes possible a particularly effective gene therapy.

In another aspect, the invention provides a mammalian cell comprising an exogenous oligoribonucleotide, wherein the oligoribonucleotide has a double stranded structure (dsRNA) comprising two separate RNA strands, wherein one strand of the dsRNA has a region I which is complementary to an RNA transcript of at least a part of the target gene.

In another aspect, the invention provides an oligoribonucleotide having a double stranded structure (dsRNA), comprising two separate RNA strands, wherein one strand of the dsRNA has a region I which is complementary to an RNA transcript of at least a part of a target gene, wherein the region is not more than 49 nucleotides in length, and wherein the target gene is a mammalian gene.

In one embodiment of the invention, the dsRNA comprises a 3' overhang. Typically, the 3' overhang is a single nucleotide overhang.

With regards to advantageous embodiments of the medicament and of the use, reference is made to the description of the above features.

Use examples of the invention are illustrated in greater detail hereinbelow with reference to the figures, in which:

Fig. 1 shows the schematic representation of a plasmid for the *in vitro* transcription with T7- and SP6-polymerase,

Fig. 2 shows RNA following electrophoresis on an 8% polyacrylamide gel and staining with ethidium bromide,

Fig. 3 shows a representation of radioactive RNA transcripts following electrophoresis on an 8% polyacrylamide gel with 7 M urea by means of an instant imager, and

Figs. 4a - e show Texas Red and YFP fluorescence in murine fibroblasts.

Use example 1:

The inhibition of transcription was detected by means of sequence homologous dsRNA in an *in vitro* transcription system with a nuclear extract from human HeLa cells. The DNA template for this experiment was plasmid pCMV1200 which had been linearized by means of *Bam*HI.

Generation of the template plasmids:

The plasmid shown in fig. 1 was constructed for use in the enzymatic synthesis of the dsRNA. To this end, a polymerase chain reaction (PCR) with the "positive control DNA" of the HelaScribe® Nuclear Extract *in vitro* transcription kit by Promega, Madison, USA, as

DNA template was first carried out. One of the primers used contained the sequence of an *Eco*RI cleavage site and of the T7 RNA polymerase promoter as shown in sequence listing No. 1. The other primer contained the sequence of a *Bam*HI cleavage site and of the SP6 RNA polymerase promoter as shown in sequence listing No. 2. In addition, the two primers had, at the 3' ends, regions which were identical with or complementary to the DNA template. The PCR was carried out by means of the "*Taq PCR Core Kits*" by Qiagen, Hilden, Germany, following the manufacturer's instructions. 1.5 mM $MgCl_2$, in each case 200 µM dNTP, in each case 0.5 µM primer, 2.5 U *Taq* DNA polymerase and approximately 100 ng of "positive control DNA" were employed as template in PCR buffer in a volume of 100 µl. After initial denaturation of the template DNA by heating for 5 minutes at 94°C, amplification was carried out in 30 cycles of denaturation for in each case 60 seconds at 94°C, annealing for 60 seconds at 5°C below the calculated melting point of the primers and polymerization for 1.5-2 minutes at 72°C. After a final polymerization of 5 minutes at 72°C, 5 µl of the reaction were analyzed by agarose-gel electrophoresis. The length of the DNA fragment amplified thus was 400 base pairs, 340 base pairs corresponding to the "positive control DNA". The PCR product was purified, hydrolyzed with *Eco*RI and *Bam*HI and, after repurification, employed in the ligation together with a pUC18 vector which had also been hydrolyzed by *Eco*RI and *Bam*HI. *E. coli* XL1-blue was then transformed. The plasmid obtained (pCMV5) carries a DNA fragment whose 5' end is flanked by the T7 promoter and whose 3' end is flanked by the SP6 promoter. By linearizing the plasmid with *Bam*HI, it can be employed *in vitro* with the T7-RNA polymerase for the run-off transcription of a single-stranded RNA which is 340 nucleotides in length and shown in sequence listing No. 3. If the plasmid is linearized with *Eco*RI, it can be employed for the run-off transcription with SP6 RNA polymerase,

giving rise to the complementary strand. In accordance with the method outlined hereinabove, an RNA 23 nucleotides in length was also synthesized. To this end, a DNA shown in sequence listing No. 4 was ligated with the pUC18 vector via the *Eco*RI and *Bam*HI cleavage sites.

Plasmid pCMV1200 was constructed as DNA template for the *in-vitro* transcription with HeLa nuclear extract. To this end, a 1 191 bp EcoRI/BamHI fragment of the positive control DNA contained in the HeLaScribe® Nuclear Extract *in vitro* transcription kit was amplified by means of PCR. The amplified fragment encompasses the 828 bp "immediate early" CMV promoter and a 363 bp transcribable DNA fragment. The PCR product was ligated to the vector pGEM-T via "T-overhang" ligation. A BamHI cleavage site is located at the 5' end of the fragment. The plasmid was linearized by hydrolysis with BamHI and used as template in the run-off transcription.

In-vitro transcription of the complementary single strands:

pCMV5 plasmid DNA was linearized with *Eco*RI or *Bam*HI. It was used as DNA template for an *in-vitro* transcription of the complementary RNA single strands with SP6 and T7 RNA polymerase, respectively. The "Riboprobe *in vitro* Transcription" system by Promega, Madison, USA, was employed for this purpose. Following the manufacturer's instructions, 2 µg of linearized plasmid DNA were incubated in 100 µl of transcription buffer and 40 U T7 or SP6 RNA polymerase for 5-6 hours at 37°C. The DNA template was subsequently degraded by addition of 2.5 µl of RNase-free DNase RQ1 and incubation for 30 minutes at 37°C. The transcription reaction was made up to 300 µl with H_2O and purified by phenol extraction. The RNA was precipitated by addition of 150 µl of 7 M ammonium acatate [sic] and 1 125 µl of

ethanol and stored at -65°C until used for the hybridization.

Generation of the RNA double strands:

For the hybridization, 500 µl of the single-stranded RNA which had been stored in ethanol and precipitated were spun down. The resulting pellet was dried and taken up in 30 µl of PIPES buffer, pH 6.4 in the presence of 80% formamide, 400 mM NaCl and 1 mM EDTA. In each case 15 µl of the complementary single strands were combined and heated for 10 minutes at 85°C. The reactions were subsequently incubated overnight at 50°C and cooled to room temperature.

Only approximately equimolar amounts of the two single strands were employed in the hybridization. This is why the dsRNA preparations contained single-stranded RNA (ssRNA) as contaminant. In order to remove these ssRNA contaminants, the reactions were treated, after hybridization, with the single-strand-specific ribonucleases bovine pancreatic RNase A and *Aspergillus oryzae* RNase T1. RNase A is an endoribonuclease which is specific for pyrimidines. RNase T1 is an endoribonuclease which preferentially cleaves at the 3' side of guanosines. dsRNA is no substrate for these ribonucleases. For the RNase treatment, the reactions in 300 µl of Tris, pH 7.4, 300 mM NaCl and 5 mM EDTA were treated with 1.2 µl of RNaseA at a concentration of 10 mg/ml and 2 µl of RNaseT1 at a concentration of 290 µg/ml. The reactions were incubated for 1.5 hours at 30°C. Thereupon, the RNases were denatured by addition of 5 µl of proteinase K at a concentration of 20 mg/ml and 10 µl of 20% SDS and incubation for 30 minutes at 37°C. The dsRNA was purified by phenol extraction and precipitated with ethanol. To verify the completeness of the RNase digestion, two control reactions were treated with ssRNA analogously to the hybridization reactions.

The dried pellet was taken up in 15 µl of TE buffer, pH 6.5, and subjected to native polyacrylamide gel electrophoresis on an 8% gel. The acrylamide gel was subsequently stained in an ethidium bromide solution and washed in a water bath. Fig. 2 shows the RNA which had been visualized in a UV transilluminator. The *sense* RNA which had been applied to lane 1 and the *antisense* RNA which had been applied to lane 2 showed a different migration behavior under the chosen conditions than the dsRNA of the hybridization reaction which had been applied to lane 3. The RNase-treated *sense* RNA and *antisense* RNA which had been applied to lanes 4 and 5, respectively, produced no visible band. This shows that the single-stranded RNAs had been degraded completely. The RNase-treated dsRNA of the hybridization reaction which had been applied to lane 6 is resistant to RNase treatment. The band which migrates faster in the native gel in comparison with the dsRNA applied to lane 3 results from dsRNA which is free from ssRNA. In addition to the dominant main band, weaker bands which migrate faster are observed after the RNase treatment.

In-vitro transcription test with human nuclear extract:

Using the HeLaScribe® Nuclear Extract *in vitro* transcription kit by Promega, Madison, USA, the transcription efficiency of the abovementioned DNA fragment which is present in plasmid pCMV1200 and homologous to the "positive control DNA" was determined in the presence of the dsRNA (dsRNA-CMV5) with sequence homology. Also, the effect of the dsRNA without sequence homology, which corresponds to the yellow fluorescent protein (YFP) gene (dsRNA-YRP), was studied. This dsRNA had been generated analogously to the dsRNA with sequence homology. The sequence of a strand of this dsRNA can be found in sequence listing No. 5. Plasmid pCMV1200 was used as template for the run-off transcription. It carries the "immediate early" cytomegalovirus promoter which is recognized by the eukaryotic RNA polymerase II, and a transcribable DNA

fragment. Transcription was carried out by means of the HeLa nuclear extract, which contains all the proteins which are necessary for transcription. By addition of [·-^{32}P]rGTP to the transcription reaction, radiolabeled transcript was obtained. The [·-^{32}P]rGTP used had a specific activity of 400 Ci/mmol, 10 mCi/ml. 3 mM $MgCl_2$, in each case 400 µM rATP, rCTP, rUTP, 16 µM rGTP, 0.4 µM [·-^{32}P]rGTP and depending on the experiment 1 fmol of linearized plasmid DNA and various amounts of dsRNA in transcription buffer were employed per reaction. Each batch was made up to a volume of 8.5 µl with H_2O. The reactions were mixed carefully. To start the transcription, 4 U HeLa nuclear extract in a volume of 4 µl were added and incubated for 60 minutes at 30°C. The reaction was stopped by addition of 87.5 µl of quench mix which had been warmed to 30°C. To remove the proteins, the reactions were treated with 100 µl of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) saturated with TE buffer, pH 5.0, and the reactions were mixed vigorously for 1 minute. For phase separation, the reactions were spun for approximately 1 minute at 12 000 rpm and the top phase was transferred into a fresh reaction vessel. Each reaction was treated with 250 µl of ethanol. The reactions were mixed thoroughly and incubated for at least 15 minutes on dry ice/methanol. To precipitate the RNA, the reactions were spun for 20 minutes at 12 000 rpm and 40°C. The supernatant was discarded. The pellet was dried in vacuo for 15 minutes and resuspended in 10 µl of H_2O. Each reaction was treated with 10 µl of denaturing loading buffer. The free GTP was separated from the transcript formed by means of denaturing polyacrylamide gel electrophoresis on an 8% gel with 7 M urea. The RNA transcripts formed upon transcription with HeLa nuclear extract, in denaturing loading buffer, were heated for 10 minutes at 90°C and 10 µl aliquots were applied immediately to the freshly washed pockets. The electrophoresis was run at 40 mA. The amount of the radioactive ssRNA formed upon

transcription was analyzed after electrophoresis with the aid of an *Instant Imager*.

Fig. 3 shows the radioactive RNA from a representative test, shown by means of the *Instant Imager*. Samples obtained from the following transcription reactions were applied:

Lane 1: without template DNA, without dsRNA;
Lane 1: 50 ng of template DNA, without dsRNA;
Lane 3: 50 ng of template DNA, 0.5 µg of dsRNA YFP;
Lane 4: 50 ng of template DNA, 1.5 µg of dsRNA YFP;
Lane 5: 50 ng of template DNA, 3 µg of dsRNA YFP;
Lane 6: 50 ng of template DNA, 5 µg of dsRNA YFP;
Lane 7: without template DNA, 1.5 dsRNA YFP;
Lane 8: 50 ng of template DNA, without dsRNA;
Lane 9: 50 ng of template DNA, 0.5 µg of dsRNA CMV5;
Lane 10: 50 ng of template DNA, 1.5 µg of dsRNA CMV5;
Lane 11: 50 ng of template DNA, 3 µg of dsRNA CMV5;
Lane 12: 50 ng of template DNA, 5 µg of dsRNA CMV5;

It emerged that the amount of transcript was reduced markedly in the presence of dsRNA with sequence homology in comparison with the control reaction without dsRNA and with the reactions with dsRNA YFP without sequence homology. The positive control in lane 2 shows that radioactive transcript was formed upon the *in-vitro* transcription with HeLa nuclear extract. The reaction is used for comparison with the transcription reactions which had been incubated in the presence of dsRNA. Lanes 3 to 6 show that the addition of non-sequentially-specific dsRNA YFP had no effect on the amount of transcript formed. Lanes 9 to 12 show that the addition of an amount of between 1.5 and 3 µg of sequentially-specific dsRNA CMV5 leads to a reduction in the amount of transcript formed. In order to exclude that the effects observed are based not on the dsRNA but on any contamination which might have been carried along accidentally during the preparation of the dsRNA,

a further control was carried out. Single-stranded RNA was transcribed as described above and subsequently subjected to the RNase treatment. It was demonstrated by means of native polyacrylamide gel electrophoresis that the ssRNA had been degraded completely. This reaction was subjected to phenol extraction and ethanol precipitation and subsequently taken up in PE buffer, as were the hybridization reactions. This gave a sample which contained no RNA but had been treated with the same enzymes and buffers as the dsRNA. Lane 8 shows that the addition of this sample had no effect on transcription. The reduction of the transcript upon addition of sequence-specific dsRNA can therefore be ascribed unequivocally to the dsRNA itself. The reduction of the amount of transcript of a gene in the presence of dsRNA in a human transcription system indicates an inhibition of the expression of the gene in question. This effect can be attributed to a novel mechanism caused by the dsRNA.

Use example 2:

The test system used for these *in-vivo* experiments was the murine fibroblast cell line NIH3T3, ATCC CRL-1658. The YFP gene was introduced into the nuclei with the aid of microinjection. Expression of YFP was studied under the effect of simultaneously cotransfected dsRNA with sequence homology. This dsRNA YFP shows homology with the 5'-region of the YFP gene over a length of 315 bp. The nucleotide sequence of a strand of the dsRNA YRP is shown in sequence listing No. 5. Evaluation under the fluorescence microscope was carried out 3 hours after injection with reference to the greenish-yellow fluorescence of the YFP formed.

Construction of the template plasmid, and preparation of the dsRNA:

A plasmid was constructed following the same principle as described in use example 1 to act as template for the production of the YFP dsRNA by means of T7 and SP6

in-vitro transcription. Using the primer *Eco*_T7_YFP as shown in sequence listing No. 6 and *Bam*_SP6_YFP as shown in sequence listing No. 7, the desired gene fragment was amplified by PCR and used analogously to the above description for preparing the dsRNA. The dsRNA YFP obtained is identical to the dsRNA used in use example 1 as non-sequence-specific control.

A dsRNA linked chemically at the 3' end of the RNA as shown in sequence listing No. 8 to the 5' end of the complementary RNA via a C18 linker group was prepared (L-dsRNA). To this end, synthons modified by disulfide bridges were used. The 3'-terminal synthon is bound to the solid support via the 3' carbon with an aliphatic linker group via a disulfide bridge. In the 5'-terminal synthon of the complementary oligoribonucleotide which is complementary to the 3'-terminal synthon of the one oligoribonucleotide, the 5'-trityl protecting group is bound via a further aliphatic linker and a disulfide bridge. Following synthesis of the two single strands, removal of the protecting groups and hybridization of the complementary oligoribonucleotides, the thiol groups which form are brought into spatial vicinity. The single strands are linked to each other by oxidation via their aliphatic linkers and a disulfide bridge. This is followed by purification with the aid of HPLC.

Preparation of the cell cultures:

The cells were incubated in DMEM supplemented with 4.5 g/l glucose, 10% fetal bovine serum in culture dishes at 37°C under a 7.5% CO_2 atmosphere and passaged before reaching confluence. The cells were detached with trypsin/EDTA. To prepare for microinjection, the cells were transferred into Petri dishes and incubated further until microcolonies formed.

Microinjection:

For the microinjection, the culture dishes were removed from the incubator for approximately 10 minutes. Approximately 50 nuclei were injected singly per reaction within a marked area using the AIS microinjection system from Carl Zeiss, Göttingen, Germany. The cells were subsequently incubated for three more hours. For the microinjection, borosilicate glass capillaries from Hilgenberg GmbH, Malsfeld, Germany, with a diameter of less than 0.5 µm at the tip were prepared. The microinjection was carried out using a micromanipulator from Narishige Scientific Instrument Lab., Tokyo, Japan. The injection time was 0.8 seconds and the pressure was approximately 100 hPa. The transfection was carried out using the plasmid pCDNA YFP, which contains an approximately 800 bp *Bam*HI/*Eco*RI fragment with the YFP gene in vector pcDNA3. The samples injected into the nuclei contained 0.01 µg/µl of pCDNA-YFP and Texas Red coupled to dextran-70000 in 14 mM NaCl, 3 mM KCl, 10 mM KPO_4 [sic], ph 7.5. Approximately 100 pl of RNA with a concentration of 1 µM or, in the case of the L-dsRNA, 375 µM were additionally added.

The cells were studied under a fluorescence microscope with excitation with the light of the excitation wavelength of Texas Red, 568 nm, or of YFP, 488 nm. Individual cells were documented by means of a digital camers. Figures 4a-e show the result for NIH3T3 cells. In the cells shown in Fig. 4a, *sense*-YFP-ssRNA has been injected, in Fig. 4b *antisense*-YFP-ssRNA, in Fig. 4c dsRNA-YFP, in Fig. 4d no RNA and in Fig. 4e L-dsRNA.

The field on the left shows in each case the fluorescence of cells with excitation at 568 nm. The fluorescence of the same cells at an excitation of 488 nm is seen on the right. The Texas Red fluorescence of all the cells shown demonstrates that the injection solution had been applied successfully into the nuclei

and that cells with successful hits were still alive after three hours. Dead cells no longer showed Texas Red fluorescence.

The right fields of each of figures 4a and 4b show that YFP expression was not visibly inhibited when the single-stranded RNA was injected into the nuclei. The right field of Fig. 4c shows cells whose YFP fluorescence was no longer detectable after the injection of dsRNA-YFP. Fig. 4d shows cells into which no RNA had been injected, as control. The cell shown in fig. 4e shows YFP fluorescence which can no longer be detected owing to the injection of the L-dsRNA which shows regions with sequence homology to the YFP gene. This result demonstrates that even shorter dsRNAs can be used for specifically inhibiting gene expression in mammals when the double strands are stabilized by chemically linking the single strands.

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition or further features in various embodiments of the invention.

It is to be understood that a reference herein to a prior art publication does not constitute an admission that the publication forms a part of the common general knowledge in the art in Australia, or any other country.

EDITORIAL NOTE

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There is no page 20

Literatur:

Asanuma, H., Ito, T., Yoshida, T., Liang, X. & Komiyama, M. (1999). Photoregulation der Bildung und Dissoziation eines DNA-Duplexes durch *cis-trans*-Isomerisierung einer Azobenzoleinheit. *Angew. Chem.* **111**, 2547-2549.

Azhayeva, E., Azhayev, A., Auriola, S., Tengvall, U., Urtti, A. & Lönnberg, H. (1997). Inhibitory properties of double helix forming circular oligonucleotides. *Nucl. Acids Res.* **25**, 4954-4961.

Castelli, J., Wood, K.A. & Youle, R.J. (1998). The 2-5A system in viral infection and apoptosis. *Biomed. Pharmacother.* **52**, 386-390.

Dolinnaya, N.G., Blumenfeld, M., Merenkova, I., Oretskaya, T.S., Krynetskaya, N.F., Ivanovskaya, M.G., Vasseur, M. & Shabarova, Z.A. (1993). Oligonucleotide circularization by template-directed chemical ligation. *Nucl. Acids Res.* **21**, 5403-5407.

Expert-Bezancon, A., Milet, M. & Carbon, P. (1983). Precise localization of several covalent RNA-RNA cross-link in *Escherichia coli* 16S RNA. *Eur. J. Biochem.* **136**, 267-274.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. & Mello, C.C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Gao, H., Yang, M., Patel, R. & Cook, A.F. (1995). Circulaization of oligonucleotides by disulfide bridge formation. *Nucl. Acids Res.* **23**, 2025-2029.

Gryaznov, S.M. & Letsinger, R.L. (1993). Template controlled coupling and recombination of oligonucleotide blocks containing thiophosphoryl groups. *Nucl. Acids Res.* **21**, 1403-1408.

Kaufman, R.J. (1999). Double-stranded RNA-activated protein kinase mediates virus-induced apoptosis: A new role for an old actor. *Proc. Natl. Acad. Sci. USA* **96**, 11693-11695.

Lipson, S.E. & Hearst, J.E. (1988). Psoralen cross-linking of ribosomal RNA. In *Methods in Enzymology* Anonymous pp. 330-341.

Liu, Z.R., Sargueil, B. & Smith, C.W. (1998). Detection of a novel ATP-dependent cross-linked protein at the 5' splice site-U1 small nuclear RNA duplex by methylene blue-mediated photo-cross-linking. *Mol. Cell. Biol.* **18**, 6910-6920.

Micura, R. (1999). Cyclic oligoribonucleotides (RNA) by solid-phase synthesis. *Chem. Eur. J.* **5**, 2077-2082.

Skripkin, E., Isel, C., Marquet, R., Ehresmann, B. & Ehresmann, C. (1996). Psoralen crosslinking between human immunodeficiency virus type 1 RNA and primer $tRNA_3^{Lys}$. *Nucl. Acids Res.* **24**, 509-514.

Wang, S. & Kool, E.T. (1994). Circular RNA oligonucleotides. Synthesis, nucleic acid binding properties, and a comparison with circular DNAs. *Nucl. Acids Res.* **22**, 2326-2333.

Wang, Z. & Rana, T.M. (1996). RNA conformation in the Tat-TAR complex determined by site-specific photo-cross-linking. *Biochem.* **35**, 6491-6499.

Watkins, K.P. & Agabian, N. (1991). *In vivo* UV cross-linking of U snRNAs that paticipate in trypanosome trans-splicing. *Genes & Development* **5**, 1859-1869.

Wengel, J. (1999). Synthesis of 3'-*C*- and 4'-*C*-branched oligodeoxynucleotides and the development of locked nucleic acid (LNA). *Acc. Chem. Res.* **32**, 301-310.

Zwieb, C., Ross, A., Rinke, J., Meinke, M. & Brimacombe, R. (1978). Evidence for RNA-RNA cross-link formation in *Escherichia coli* ribosomes. *Nucl. Acids Res.* **5**, 2705-2720.

EDITORIAL NOTE

APPLICATION NUMBER – 32713/00

The following Sequence Listing pages 1 to 4 are part of the description. The claims pages follow on pages "24" to "44".

Sequence Listing

<110> Kreutzer Dr., Roland
Limmer Dr., Stephan

<120> Method and medicament for inhibiting the expression of a given gene

<130> 400968

<140>
<141>

<150> 199 03 713.2
<151> 1999-01-30

<150> 199 56 568.6
<151> 1999-11-24

<160> 8

<170> PatentIn Ver. 2.1

<210> 1
<211> 45
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: EcoRI cleavage site, T7 RNA Polymerase promoter

<400> 1
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<210> 2

<211> 50
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: BamHI cleavage site, SP6 RNA Polymerase promoter

<400> 2
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<210> 3
<211> 340
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: RNA which corresponds to a sequence from the positive control DNA of the HeLa Nuclear Extract in vitro transcription kit from Promega

<400> 3
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gcaccgugua ugaaaucuaa caaugcgccc aucgucaucc ucggcaccgu cacccuggau 120
gcuguaggca uaggcuuggu uaugccggua cugccgggcc ucuugcggga uaucguccau 180
uccgacagca ucgccaguca cuauggcgug cugcuagcgc uauaugcguu gaugcaauuu 240
cuaugcgcac ccguucucgg agcacugucc gaccgcuuug gccgccgccc aguccugcuc 300
gcuucgcuac uuggagccac uaucgacuac gcgaucaugg 340

<210> 4
<211> 363
<212> DNA
<213> Artificial Sequence

<220>

<223> Description of the artificial sequence:
DNA which corresponds to a sequence from the positive control DNA of the HeLa Nuclear Extract in vitro transcription kit from Promega

<400> 4
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gcaccgtgta tgaaatctaa caatgcgctc atcgtcatcc tcggcaccgt caccctggat 120
gctgtaggca taggcttggt tatgccggta ctgccgggcc tcttgcgggg tatcgtccat 180
tccgacagca tcgccagtca ctatggcgtg ctgctagcgc tatatgcgtt gatgcaattt 240
ctatgcgcac ccgttctcgg agcactgtcc gaccgctttg gccgccgccc agtcctgctc 300
gcttcgctac ttggagccac tatcgactac gcgatcatgg cgaccacacc cgtcctgtgg 360
atc 363

<210> 5
<211> 315
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence:
Sequence from the YFP gene

<400> 5
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ggcgacguaa acggccacaa guucagcgug uccggcgagg gcgagggcga ugccaccuac 120
ggcaagcuga cccugaaguu caucugcacc accggcaagc ugcccgugcc cuggcccacc 180
cucgugacca cccugaccua cggcgugcag ugcuucagcc gcuaccccga ccacaugaag 240
cagcacgacu ucuucaaguc cgccaugccc gaaggcuacg uccaggagcg caccaucuuc 300
uucaaggacg acggc 315

<210> 6
<211> 52
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence:

EcoRI cleavage site, T7 RNA Polymerase promoter, complementary region to the YFP gene

<400> 6
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<210> 7
<211> 53
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: BamHI cleavage site, SP6 RNA Polymerase promoter, complementary region to the YFP gene

<400> 7
gggatccatt taggtgacac tatagaatac gccgtcgtcc ttgaagaaga tgg 53

<210> 8
<211> 21
<212> RNA
<213> Artificial Sequence

<220>
<223> Description of the artificial sequence: RNA which corresponds to a sequence from the YFP gene

<400> 8
ucgagcugga cggcgacgua a 21

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A method for inhibiting the expression of a given target gene in a cell, wherein an oligoribonucleotide with double-stranded structure (dsRNA) which has 15 to 49 base pairs is introduced into the cell, wherein one strand of the dsRNA has a region I with not more than 49 successive nucleotide pairs and which is at least in parts complementary to the target gene and wherein a complementary region II within the double-stranded structure is formed by two separate RNA single strands.

2. The method according to claim 1, wherein the dsRNA is enclosed by micellar structures.

3. The method according to claim 2, wherein the dsRNA is enclosed by liposomes.

4. The method according to any one of the preceding claims, wherein the dsRNA is enclosed by natural viral capsids or by chemically or enzymatically produced artificial capsids or structures derived therefrom.

5. The method according to any one of the preceding claims, wherein the target gene is expressed in eukaryotic cells.

6. The method according to any one of the preceding claims, wherein the target gene is selected from the following group: oncogene, cytokine gene, Id-protein gene, development gene, prion gene.

7. The method according to any one of the preceding claims, wherein the target gene is expressed in pathogenic organisms.

8. The method according to claim 7, wherein the pathogenic organism is plasmodia.

9. The method according to any one of the preceding claims, wherein the target gene is part of a virus or viroid.

10. The method according to claim 9, wherein the virus is a virus or viroid which is pathogenic for humans.

11. The method according to claim 9, wherein the virus or viroid is a virus or viroid which is pathogenic for animals or phytopathogenic.

12. The method according to any one of the preceding claims, wherein segments of the dsRNA are in double-stranded form.

13. The method according to any one of the preceding claims, wherein the ends of the dsRNA are modified in order to counteract degradation in the cell or dissociation into the single strands.

14. The method according to any one of the preceding claims, wherein the cohesion of the complementary region II, which is caused by the nucleotide pairs, is increased by at least one further chemical linkage.

15. The method of claim 14, wherein the cohesion of the complementary region II is increased by two further chemical linkages.

16. The method according to claim 14 or 15, wherein the chemical linkage is formed by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, van-der-Waals or stacking interactions, or by metal-ion coordination.

17. The method of claim 16, wherein the chemical linkage is formed by van-der-Waals or stacking interactions.

18. The method according to claims 14 to 17, wherein the chemical linkage is generated at at least one end of the complementary region II.

19. The method according to claim 18 wherein the chemical linkage is generated at both ends of the complementary region II.

20. The method according to any one of claims 14 to 19, wherein the chemical linkage is formed by means of one or more compound groups.

21. The method according to claim 20, wherein the compound groups are poly(oxyphosphinicooxyl-1,3-propanediol) and/or polyethylene glycol chains.

22. The method according to any one of claims 14 to 21, wherein the chemical linkage is formed by purine analogs used in the complementary regions II in place of purines.

23. The method according to any one of claims 14 to 22, wherein the chemical linkage is formed by azabenzene unit introduced into the complementary regions II.

24. The method according to any one of claims 14 to 23, wherein the chemical linkage is formed by branched nucleotide analogs used in the complementary regions II in place of nucleotides.

25. The method according to any one of claims 14 to 24, wherein at least one of the following groups is used for generating the chemical linkage: methylene blue; bifunctional groups, N-acetyl-N'-(p-glyoxyl-benzoyl)cystamine; 4-thiouracil; psoralene.

26. The method of claim 25 wherein the bifunctional group is bis(2-chloroethyl)amine.

27. The method according to any one of claims 14 to 26, wherein the chemical linkage is formed by thiophosphoryl groups provided at the ends of the double-stranded region.

28. The method according to any one of claims 14 to 27, wherein the chemical linkage at the ends of the double-stranded region is formed by triple-helix bonds.

29. The method according to any one of the preceding claim, wherein at least one 2'-hydroxyl group of the nucleotides of the dsRNA in the complementary region II is replaced by a chemical group.

30. The method of claim 29, wherein the chemical group is a 2'-amino or a 2'-methyl group.

31. The method according to any one of the preceding claims, wherein at least one nucleotide in at least one strand of the complementary region II is a "locked nucleotide" with a sugar ring which is chemically modified.

32. The method of claim 31, wherein the "locked nucleotide" with a sugar ring is chemically modified by a 2'-O, 4'-C-methylene bridge.

33. The method according to any one of the preceding claims, wherein the dsRNA is bound to, associated with or surrounded by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically.

34. The method according to claim 33, wherein the coat protein is derived from polyomavirus.

35. The method according to claim 33 or 34, wherein the coat protein contains the polyomavirus protein 1 (VP1) and/or virus protein 2 (VP2).

36. The method according to any one of claims 33 to 35, wherein when a capsid or capsid-type structure is formed from the coat protein, one side faces the interior of the capsid or capsid-type structure.

37. The method according to any one of the preceding claims, wherein the dsRNA is complementary to the primary or processed RNA transcript of the target gene.

38. The method according to any one of the preceding claims, wherein the cell is a vertebrate cell or a human cell.

39. The method according to any one of the preceding claims, wherein at least two dsRNAs which differ from each other are introduced into the cell, wherein at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes.

40. The method according to any one of the preceding claims, wherein one of the target genes is the PKR gene.

41. A medicament with at least one oligoribonucleotide with double-stranded structure (dsRNA) which contains 15 to 49 base pairs for inhibiting the expression of a given target gene in mammalian cells, wherein one strand of the dsRNA has a region I with not more than 49 successive nucleotide pairs and which is at least in parts complementary to the target gene and where a complementary region II within the double-stranded structure is formed by two separate RNA single strands.

42. The method according to claim 41, wherein the dsRNA is enclosed by micellar structures.

43. The medicament of claim 42 wherein the micellar structures are liposomes.

44. The medicament according to any one of claims 41 to 43, wherein the dsRNA is enclosed by natural viral capsids

or by chemically or enzymatically produced artificial capsids or structures derived therefrom.

45. The medicament according to any one of claims 41 to 44, wherein the target gene can be expressed in eukaryotic cells.

46. The medicament according to any one of claims 41 to 45, wherein the target gene is selected from the following group: oncogene, cytokine gene, Id-protein gene, development gene, prion gene.

47. The medicament according to any one of claims 41 to 46, wherein the target gene can be expressed in pathogenic organisms.

48. The medicament of claim 47 wherein the pathogenic organism is plasmodia.

49. The medicament accord9ng to any one of claims 41 to 48, wherein the target gene is part of a virus or viroid.

50. The medicament according to claim 49, wherein the virus is a virus or viroid which is pathogenic for humans.

51. The medicament according to claim 49, wherein the virus or viroid is a virus or viroid which is pathogenic for animals or phytopathogenic.

52. The medicament according to any one of claims 41 to 51, wherein segments of the dsRNA are in double-stranded form.

53. The medicament according to any one of claims 41 to 52, wherein the ends of the dsRNA are modified in order to counteract degradation in the cell or dissociation into the single strands.

54. The medicament according to any one of claims 41 to 53, wherein the cohesion of the complementary region II, which is caused by the nucleotide pairs, is increased by at least one further chemical linkage.

55. The medicament of claim 54, wherein the cohesion of the double-stranded structure is increased by two further chemical linkages.

56. The medicament according to claim 54 or 55, wherein the chemical linkage is formed by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, van-der-Waals or stacking interactions, or by metal-ion coordination.

57. The medicament according to claim 56, wherein the chemical linkage is formed by van-der-Waals or stacking interactions.

58. The medicament according to any one of claims 54 to 57, wherein the chemical linkage is generated at at least one end of the complementary region II.

59. The medicament according to claim 58, wherein the chemical linkage is generated at both ends of the complementary region II.

60. The medicament according to any one of claims 54 to 59, wherein the chemical linkage is formed by means of one or more compound groups.

61. The medicament according to claim 60, wherein the compound groups are poly(oxyphosphinicooxy-1,3-propanediol) and/or polyethylene glycol chains.

62. The medicament according to any one of claims 54 to 61, wherein the chemical linkage is formed by purine analogs used in the complementary regions II in place of purines.

63. The medicament according to any one of claims 54 to 61, wherein the chemical linkage is formed by azabenzene units inserted into the complementary regions II.

64. The medicament according to any one of claims 54 to 63, wherein the chemical linkage is formed by branched nucleotide analogs used in the complementary region II in place of nucleotides.

65. The medicament according to any one of claims 54 to 64, wherein at least one of the following groups is used for generating the chemical linkage: methylene blue; bifunctional groups; N-acetyl-N'-(p-glyoxybenzoyl)cystamine; 4-thiouracil; psoralene.

66. The medicament according to claim 65, wherein the bifunctional group is bis(2-chloroethyl)amine.

67. The medicament according to any one of claims 54 to 66, wherein the chemical linkage is formed by

thiophosphoryl groups provided at the ends of the double-stranded region.

68. The medicament according to any one of claims 54 to 67, wherein the chemical linkages are triple-helix bonds provided at the ends of the double-stranded region.

69. The medicament according to any one of claims 41 to 68, wherein at least one 2'-hydroxyl group of the nucleotides of the dsRNA in the complementary region II is replaced by a chemical group.

70. The medicament according to claim 69, wherein the chemical group is a 2'-amino or a 2'-methyl group.

71. The medicament according to any one of claims 41 to 70, wherein at least one nucleotide in at least one strand of the complementary region II is a "locked nucleotide" with a sugar ring which is chemically modified.

72. The medicament according to claim 71, wherein the "locked nucleotide" with a sugar ring is chemically modified by a 2'-O, 4'-C-methylene bridge.

73. The medicament according to any one of claims 41 to 72, wherein the dsRNA is bound to, associated with or surrounded by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically.

74. The medicament according to claim 73, wherein the coat protein is derived from the polyomavirus.

75. The medicament according to claim 73 or 74, wherein the coat protein contains the polyomavirus protein 1 (VP1) and/or virus protein 2 (VP2).

76. The medicament according to any one of claims 73 to 75, wherein when a capsid or capsid-type structure is formed from the coat protein, one side faces the interior of the capsid or capsid-type structure.

77. The medicament according to any one of claims 41 to 76, wherein one strand of the dsRNA is complementary to the primary or processed RNA transcript of the target gene.

78. The medicament according to any one of claims 41 to 77, wherein the cell is a human cell.

79. The medicament according to any one of claims 41 to 78, wherein at least two dsRNAs which differ from each other are contained in the medicament, wherein at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes.

80. The medicament according to claim 79, wherein one of the target genes is the PKR gene.

81. Use of an oligoribonucleotide with double-stranded structure (dsRNA) which has 15 to 49 base pairs for the preparation of a medicament for inhibiting the expression of a given target gene in mammalian cells, wherein one strand of the dsRNA has a region I with not more than 49 successive nucleotide pairs and which is at least in parts complementary to the target gene and

wherein a complementary region II within the double-stranded structure is formed by two separate RNA single strands.

82. Use according to claim 81, wherein the dsRNA is enclosed by micellar structures.

83. Use according to claim 82, wherein the micellar structures are liposomes.

84. Use according to any one of claims 81 to 83, wherein the dsRNA is enclosed by natural viral capsids or by chemically or enzymatically produced artificial capsids or structures derived therefrom.

85. Use according to any one of claims 81 to 84, wherein the target gene can be expressed in eukaryotic cells.

86. Use according to any one of claims 81 to 85, wherein the target gene is selected from the following group: oncogene, cytokine gene, Id-protein gene, development gene, prion gene.

87. Use according to any one of claims 81 to 86, wherein the target gene can be expressed in pathogenic organisms.

88. Use according to claim 87, wherein the pathogenic organism is plasmodia.

89. Use according to any one of claims 81 to 88, wherein the target gene is part of a virus or viroid.

90. Use according to claim 89, wherein the virus is a virus or viroid which is pathogenic for humans.

91. Use according to claim 89, wherein the virus or viroid is a virus or viroid which is pathogenic for animals or phytopathogenic.

92. Use according to any one of claims 81 to 91, wherein segments of the dsRNA are in double-stranded form.

93. Use according to any one of claims 81 to 92, wherein the ends of the dsRNA are modified in order to counteract degradation in the cell or dissociation into the single strands.

94. Use according to one of claims 81 to 93, wherein the cohesion of the complementary region II, which is caused by the complementary nucleotide pairs, is increased by at least one further chemical linkage.

95. Use according to claim 94, wherein the cohesion of the complementary region II is increased by two further chemical linkages.

96. Use according to claim 94 or 95, wherein the chemical linkage is formed by a covalent or ionic bond, a hydrogen bond, hydrophobic interactions, van-der-Waals or stacking interactions, or by metal-ion coordination.

97. Use according to claim 96, wherein the chemical linkage is formed by van-der-Waals or stacking interactions.

98. Use according to any one of claims 94 to 97, wherein the chemical linkage is generated at at least one end of the complementary region II.

99. Use according to claim 98, wherein the chemical linkage is generated at both ends of the complementary region II.

100. Use according to any one of claims 94 to 99, wherein the chemical linkage is formed by means of one or more compound groups.

101. Use according to claim 100, wherein the compound groups are poly(oxyphosphinicooxy-1,3-propanediol) and/or polyethylene glycol chains.

102. Use according to any one of claims 94 to 99, wherein the chemical linkage is formed by purine analogs used in the complementary regions II in -place of purines.

103. Use according to any one of claims 94 to 99, wherein the chemical linkage is formed by azabenzene units introduced into the complementary regions II.

104. Use according to any one of claims 94 to 99, wherein the chemical linkage is formed by branched nucleotide analogs used in the complementary regions II in place of nucleotides.

105. Use according to any one of claims 94 to 99, wherein at least one of the following groups is used for generating the chemical linkage: methylene blue;

bifunctional groups; N-acetyl-N'-(p-glyoxyl-benzoyl)cystamine; 4-thiouracil; psoralene.

106. Use according to claim 105, wherein the bifunctional group is bis(2-chloroethyl)amine.

107. Use according to any one of claims 94 to 99, wherein the chemical linkage is formed by thiophosphoryl groups attached to the ends of the double-stranded region.

108. Use according to any one of claims 94 to 99, wherein the chemical linkage at the ends of the double-stranded region is formed by triple-helix bonds.

109. Use according to any one of claims 81 to 108, wherein at least one 2'-hydrdoxyl group of the nucleotides of the dsRNA in the double-stranded structure is replaced by a chemical group.

110. Use according to claim 109, wherein the chemical group is a 2'-amino or a 2'-methyl group.

111. Use according to any one of claims 81 to 110, wherein at least one nucleotide in at least one strand of the complementary region II is a "locked nucleotide" with a sugar ring which is chemically modified.

112. Use according to claim 11, wherein the "locked nucleotide" with a sugar ring is chemically modified by a 2'-O, 4'-C-methylene bridge.

113. Use according to any one of claims 81 to 112, wherein the dsRNA is bound to, associated with or surrounded

by, at least one viral coat protein which originates from a virus, is derived therefrom or has been prepared synthetically.

114. Use according to claim 113, wherein the coat protein is derived from polyomavirus.

115. Use according to claim 113 or 114, wherein the coat protein contains the polyomavirus virus protein 1 (VP1) and/or virus protein 2 (VP2).

116. Use according to any one of claims 113 to 115, wherein, when a capsid or capsid-type structure is formed from the coat protein, one side faces the interior of the capsid or capsid-type structure.

117. Use according to any one of claims 81 to 116, wherein one strand of the dsRNA is complementary to the primary or processed RNA transcript of the target gene.

118. Use according to any one of claims 81 to 117, wherein the cell is a human cell.

119. Use according to any one of claims 81 to 118, wherein at least two dsRNAs which differ from each other are used, wherein at least segments of one strand of each dsRNA are complementary to in each case one of at least two different target genes.

120. Use according to claim 119, wherein one of the target gens is the PKR gene.

121. Use according to any one of claims 81 to 120, wherein the medicament is injectable into the bloodstream or into the interstitium of the organism to undergo therapy.

122. Use according to any one of claims 81 to 121, wherein the dsRNA is taken up into bacteria or microorganisms.

123. An oligoribonucleotide having a double stranded structure (dsRNA), comprising two separate RNA strands, wherein one strand of the dsRNA has a region I which is complementary to an RNA transcript of at least a part of a target gene, wherein the region is not more than 49 nucleotides in length, and wherein the target gene is a mammalian gene.

124. The oligoribonucleotide of claim 123, having a length of between 15 and 49 base pairs.

125. The oligoribonucleotide of claim 123 or 124, wherein the RNA transcript is a primary or a processed RNA.

126. The oligoribonucleotide of any one of claims 123 to 125, wherein the dsRNA comprises a linker between the two RNA strands.

127. The oligoribonucleotide of claim 126, wherein the linker is a polyethylene glycol linker.

128. The oligoribonucleotide of any one of claims 123 to 127, wherein the dsRNA is modified so as to be resistant to RNA degradation.

129. The oligoribonucleotide of any one of claims 123 to 127, wherein said dsRNA comprises a 3' overhang.

130. The oligoribonucleotide of claim 129, wherein said 3' overhang is a single nucleotide overhang.

131. The oligoribonucleotide of any one of claims 123 to 130, wherein said oligoribonucleotide is 21 or 23 nucleotides in length.

132. A composition comprising an oligoribonucleotide according to any one of claims 123 to 131.

133. The composition of claim 132, further comprising a second oligoribonucleotide, wherein said second oligoribonucleotide differs in sequence from said oligoribonucleotide.

134. A method for inhibiting the expression of a target gene in a mammalian cell, the method comprising:

(a) introducing into the cell an oligoribonucleotide having a double stranded structure (dsRNA), comprising two separate RNA strands, wherein one strand of the dsRNA has a region which is complementary to an RNA transcript of at least a part of a target gene; wherein the region is not more than 49 nucleotide in length, and

(b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of an RNA transcript of the target gene, thereby inhibiting expression of the target gene in the cell.

135. The method of claim 134, wherein the dsRNA has a length of between 15 and 49 base pairs.

136. The method of claim 134 or 135, wherein the RNA transcript is a primary or a processed RNA.

137. The method of any one of claims 134 to 136, wherein the dsRNA comprises between the two RNA strands.

138. The method of claim 137, wherein the linker is a polyethylene glycol linker.

139. The method of any one of claims 134 to 138, wherein the cell is a human cell.

140. A mammalian cell comprising an exogenous oligoribonucleotide, wherein the oligoribonucleotide has a double stranded structure (dsRNA) comprising two separate RNA strands, and wherein one strand of the dsRNA has a region I which is complementary to an RNA transcript of at least a part of a target gene.

141. The mammalian cell of claim 140, wherein the mammalian cell is a human cell.

142. The mammalian cell of claim 140 to 141, wherein the region is not more than 49 nucleotides in length.

143. The mammalian cell of any one of claims 140 to 142, wherein the dsRNA has a length of between 15 and 49 base pairs.

144. The mammalian cell of any one of claims 140 to 143, wherein the RNA transcript is a primary or processed RNA.

145. The mammalian cell of any one of claims 140 to 144, wherein the dsRNA comprises a linker between the two RNA strands.

146. The mammalian cell of any one of claims 140 to 145, wherein the linker is a polyethylene glycol linker.

147. The mammalian cell of any one of claims 140 to 146, wherein said dsRNA is modified so as to be resistant to RNA degradation.

148. The mammalian cell of any one of claims 140 to 147, wherein said dsRNA comprises a 3' overhang.

149. The mammalian cell of claim 148, wherein said 3' overhang is a single nucleotide overhang.

150. A method according to claim 1, substantially as hereinbefore described with reference to any one of the Examples.

151. A medicament according to claim 41, substantially as hereinbefore described with reference to any one of the Examples.

152. Use according to claim 81, substantially as hereinbefore described with reference to any one of the Examples.

153. An oligoribonucleotide according to claim 123, substantially as hereinbefore described with reference to any one of the Examples.

154. A mammalian cell according to claim 140, substantially as hereinbefore described with reference to any one of the Examples.

155. The method of any one of claims 1 to 40, 134 to 139, or 150, wherein said dsRNA comprises a 3'overhang.

156. The method of claim 155, wherein the 3' overhang is a single nucleotide overhang.

157. The medicament of any one of claims 41 to 90 or 151, wherein the said dsRNA comprises a 3' overhang.

158. The medicament of claim 157, wherein the 3' overhang is a single nucleotide overhang.

159. The use of any one of claims 81 to 122, wherein said dsRNA comprises a 3' overhang.

160. The use of claim 159, wherein the 3' overhang is a single nucleotide overhang.

Dated this 19th day of October 2004
ROLAND KREUTZER AND STEPHAN LIMMER
By their Patent Attorneys
GRIFFITH HACK

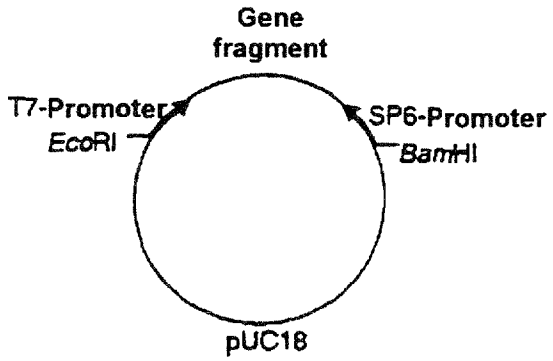

Fig. 1

Fig. 2

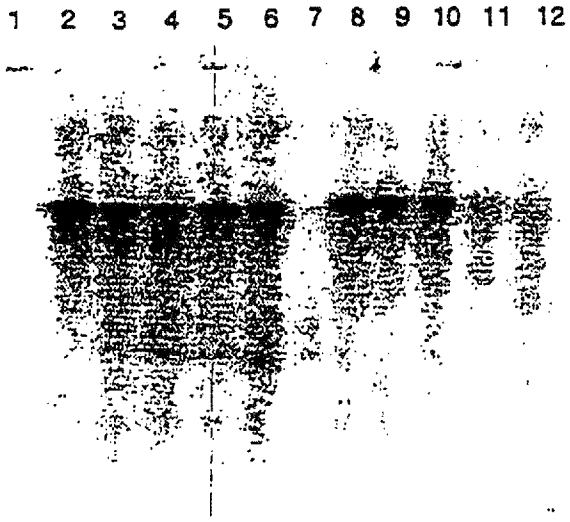

Fig. 3

Fig. 4 a

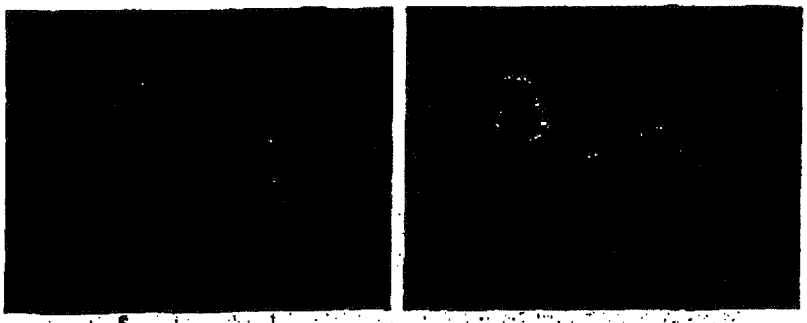

Fig. 4 b

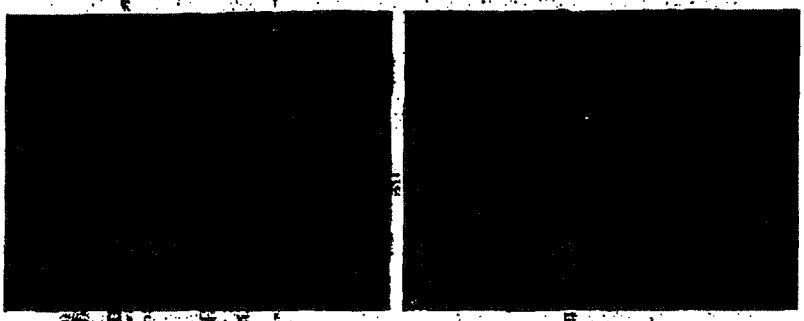

Fig. 4 c

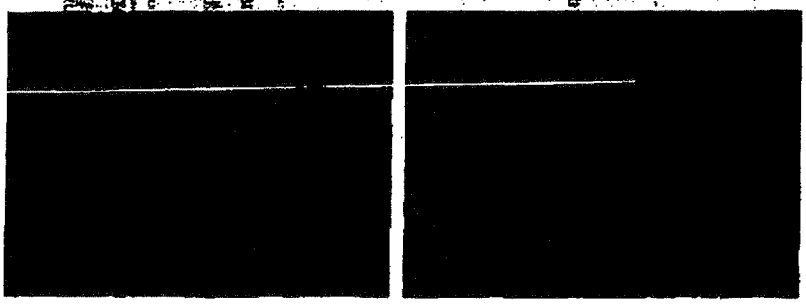

Fig. 4 d

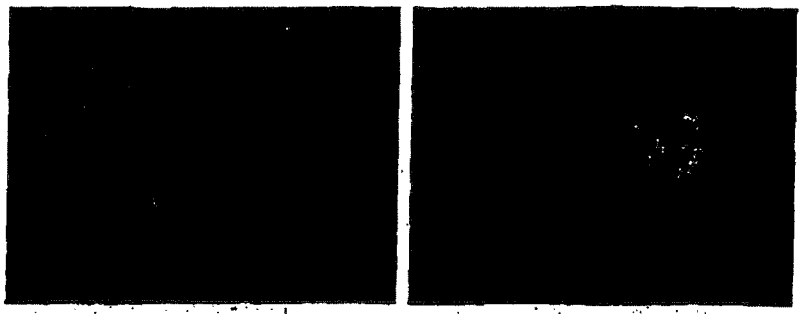

Fig. 4 e